Development and Validation of a Technique for Detection of Stress and Pregnancy in Large Whales

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LONG-TERM GOALS

To promote the use of endocrine techniques to advance our knowledge of the physiology of cetaceans and their responses to change in their environment.

OBJECTIVES

Two objectives are being nested under the overall goal of developing techniques that can be used for the detection of stress and pregnancy in large whales.

1) The first objective is to develop and conduct analytical and preliminary biological validations of pregnancy and stress hormones for three large whales (humpback whales, blue whales, and possibly insular false killer whales).

2) The second objective is to complete the biological validation using archived samples from whales with known life history or behaviors.

APPROACH

The proposal is for a two year project in which the first year will serve as the proof of concept and the second year will complete the validation and add life history and behavior data. Details of each year's activities are separated below.

Year 1 - Commercially available immunoassays previously validated in other marine mammal species will be validated for corticosterone or cortisol and progesterone in humpback and blue whale blubber. The validation will follow two forms, analytical and biological, described below.

Analytical Validation: The analytical validation will be conducted using standard methods of parallelism, accuracy and metabolite identification using high-pressure liquid chromatography (HPLC). Briefly, pooled blubber extract from animals of known gender will be serially diluted 1:2 and run as samples, in duplicate, in the assay to determine displacement by the pool. Accuracy of the assay will be determined by spiking the pool at the appropriate dilution with known amounts of hormone then performing the assays. Mass of the pool will be calculated and subtracted from the mass measured in the assay. The mean percent difference, standard error, and percent coefficient of variation, will be determined and taken as an index of accuracy of the assay. Mass added versus mass measured in the pool will be plotted in a standard scatter plot and simple regression analysis performed to determine slope, using the slope value as the index of precision of measurement of the assay. Final analytical validation of the assay will be determined through HPLC. All extracted blubber samples will be assayed in duplicate using the validated assays. Various forms of this analytical validation have been conducted over the years on a variety of biological media in our Endocrine Laboratory (Atkinson et al., In Prep; Atkinson et al., 2015; Keogh and Atkinson, 2015; Di Poi et al., 2015; Ellsworth et al., 2014; Geiger et al., 2013; Jaatinen et al., 2013; Keogh et al., 2013; Trumble et al., 2013; Seltmann et.al., 2012; Villegas-Amtmann et al., 2012; Verrier et al., 2012; Atkinson et al. 2011; Myers et al., 2009; Villegas-Amtmann et al., 2009; Nilsson et al., 2008; Petrauskas et al., 2008; Mashburn and Atkinson, 2008; Mellish et al., 2007; Mashburn and Atkinson, 2007; Greig et al., 2007; Petrauskas and Atkinson, 2006; Petrauskas et al., 2006; Mashburn and Atkinson, 2004; Oki and Atkinson, 2004; West et al., 2000; Atkinson et al., 1999).

Biological Validation: The first part of the biological validation will begin with freshly stranded whale samples from the National Marine Mammal Stranding Network or National Marine Fisheries Service (NMFS) from a humpback whale stranding (preferably in southeast Alaska). The preferred specimen will be an adult female, such that we can evaluate concentrations of corticoids and progesterone at different blubber depths as well as different locations on the body. Four locations along the axis of the whale will be assessed with both dorsal and ventrolateral samplings at each location. Each sampling will include a full depth core of blubber from the skin to the underlying muscle. Understanding the variation in concentrations at the various sites and in the various blubber depths will round out the validations so that we will have good confirmation (and confidence) that our measurements accurately reflect the biological variation that naturally occurs in any animal.

Year 2. The second year of the proposal will round out the data sets and include samples from discreet seasons (and thus geography). The locations of primary interest are the winter breeding grounds in the Hawaiian Islands and the summer feeding grounds in southeast Alaska. These samples will allow us to

assess the normal variations in endocrine profiles that can be expected at different times of year. Samples from 4 main groups of whales will be sought with the following priority:

1) adult females with calves

2) adult females that may be pregnant

3) juvenile females

4) adult males

Samples with a maximum amount of life history and behavioral knowledge will be sought over samples with less corresponding information.

WORK COMPLETED

This project has started on time and is up to date in terms of our expectations. We have nearly completed objective 1 for two species, humpback whales (*Megaptera novaeangliae*) and blue whale (*Balaenoptera musculus*), and are still waiting for a freshly stranded humpback whale to conduct multiple samples. We are currrently working on objective 2. Co-PI Jan Straley and I have accessed the first set of SPLASH samples from NMFS Southwest Fisheries Science Center. Details are provided in the results section below, based on each species.

RESULTS

a) Assay Validations for Blue Whales (Balaenoptera musculus):

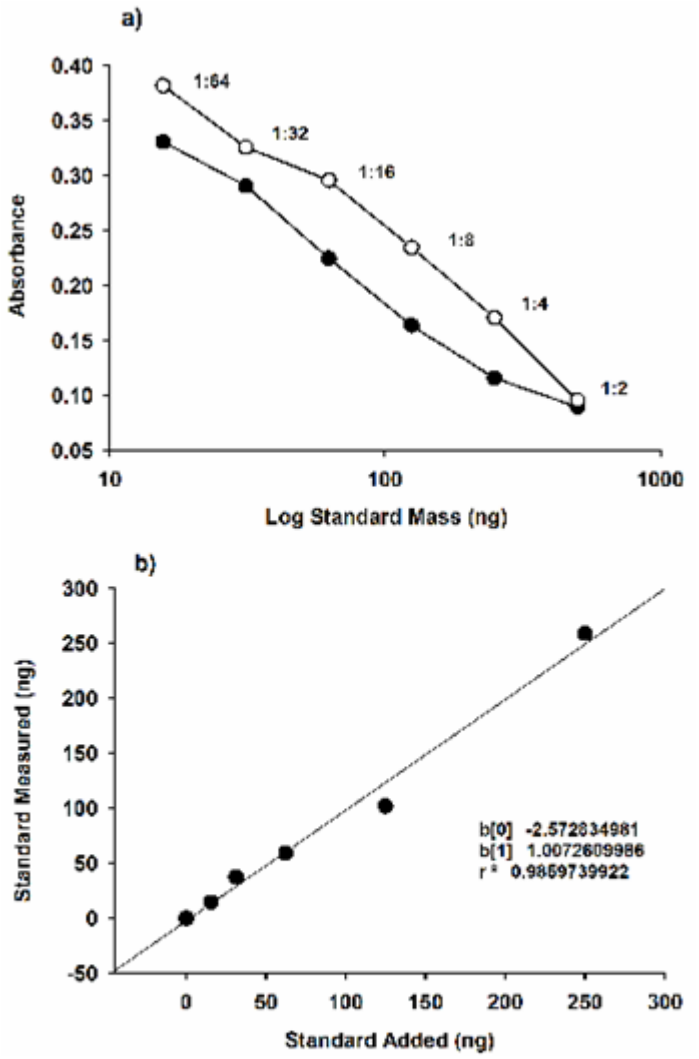

Fig 1: Enzyme immunoassay (EIA) validations for progesterone. a) Parallel displacement of the standard curve (solid circles) measured by optical absorbance, using female blue whale blubber (hollow circles). b) Accuracy of the assay with the mass added (ng) reflecting the mass measured (ng) across standard curve.

The enzyme immunoassays (EIA) for both progesterone and cortisol were validated for blue whales (Fig 1 and 2). The use of extracted blubber from female blue whales in the progesterone assay resulted in parallel displacement of the standard curve (Fig 1a). The accuracy of the mass measured from the amount added was linear (Fig 1b). Likewise with the cortisol assay, parallel displacement of the standard curve occurred with the blubber extracted from both male and female blubber pools (Fig 2a). Linearity was also achieved with the amount of cortisol added versus what was measured (Fig 2b).

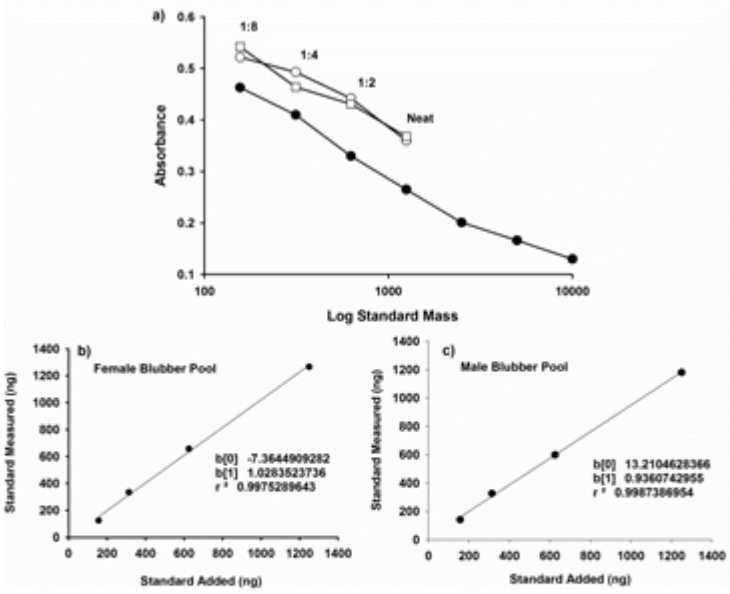

Fig 2: Enzyme immunoassay (EIA) validations for cortisol. a) Parallel displacement of the standard curve (solid circles) using both female (hollow circles) and male (hollow squares) blue whale blubber. Accuracy of the assay with the mass added (ng) reflecting the mass measured (ng) across standard curve, for b) female and c) male blue whale blubber.

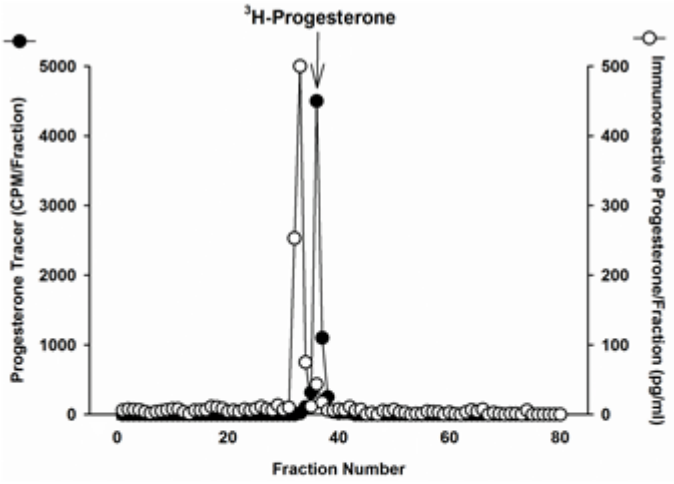

Fig 3: HPLC of progesterone in female whale blubber (hollow circles) Filled circles represent tritiated progesterone tracer. Note that 67.4% of the immunoactive progesterone (fractions 32-34) is more polar that progesterone, whereas only 4.9% overlaps with the tritiated progesterone (fractions 34-38). A 1 ml/min flow rate was used with a 32-50-75-100% acetonitrile /H_2o gradient.

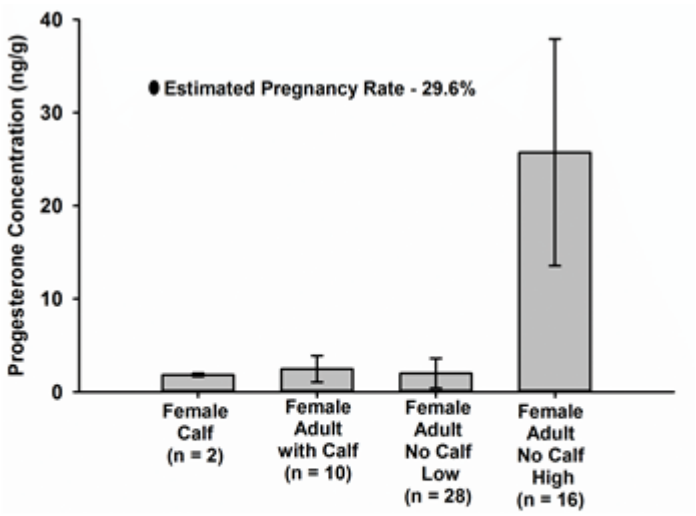

Fig 4: Mean (± sem) blubber progesterone concentrations (ng/g) from female blue whales in different reproductive states. Female whales with no calves were split into those with low (<5.83ng/g) and high (>5.83 ng/g) progesterone concentrations. Adult females with no calf and high progesterone concentrations were considered pregnant.

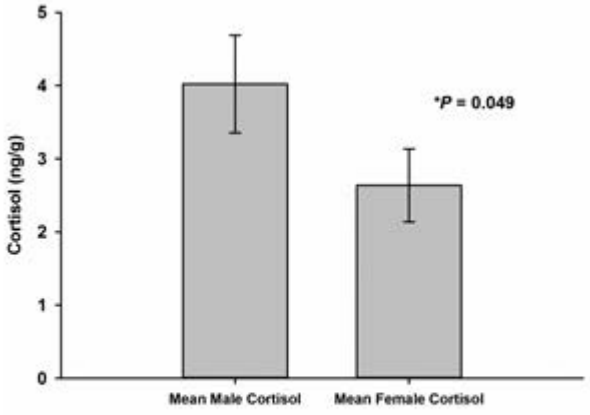

Fig 5: Mean (± sem) cortisol concentrations (ng/g) in male and female blue whale blubber.

Blubber concentrations of progesterone were low and not significantly different in female blue whale calves and adults with a calf (Fig 4). The progesterone concentrations from the adult females with a calf (2.47±1.42 ng/g x±sd) were used as the positive proof of a non-pregnant whale. Progesterone concentrations of the adult females with no calves were split into those with low and high progesterone, using the upper concentration of non-pregnant whales (>5.83 ng/g) as a cut-off. The adult females with low progesterone

The HPLC profile for ^{3}H-progestrone exhibited a clearly defined peak between fractions 34-38 (Fig 3). The female blue whale blubber had two immunoreactive peaks; the large and more polar peak occurred in fractions 32-34 and represented 67.4% of the pooled sample mass. The smaller immunoreactive peaked occurred in fractions 36-37, and represented 4.9% of the pooled sample mass (Fig 3).

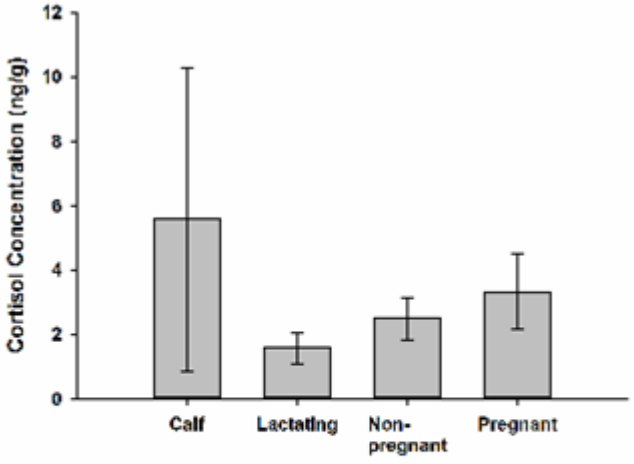

Fig 6: Mean (± sem) blubber cortisol concentrations from female blue whales in different reproductive states, female whales with calves (lactating n=10), females with no calf and low progesterone (non-pregnant, n=28) and females with no calf and high progesterone (pregnant n=16).

concentrations (2.00±1.60 ng/g) were considered non-pregnant and those with high progesterone (25.73±12.18 ng/g) were considered pregnant (Fig 4). The pregnant females had significantly higher progesterone concentrations than all other female groups ($p<0.001$). When the proportions of adult females with high progesterone were compared to the total number of adult females in the study, it equates to a 29.6% pregnancy rate (Fig 4).

Cortisol concentrations differed significantly between male and female blue whales (Fig 5). Cortisol concentrations were extremely variable in the calves (Fig 6). There were no significant differences in the cortisol concentrations in any group of female blue whales (Fig 6).

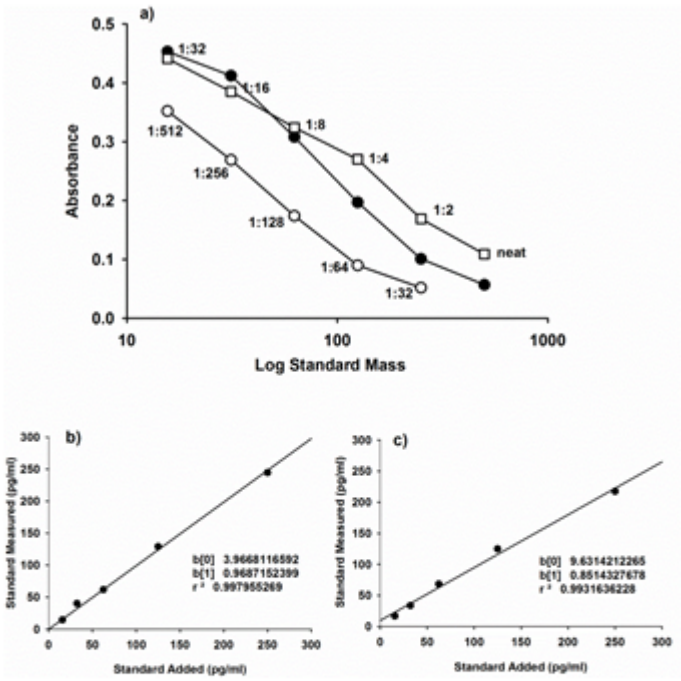

Fig 7. Enzyme immunoassay (EIA) validations for progesterone in humpback whale blubber. a) Parallel displacement of the standard curve (solid circles) measured by optical absorbance, using both female (open circles) and male (open squares) blubber. Accuracy of the assay with mass added (ng) reflecting the mass measured (ng) across the standard curve for b) female and c) male humpback whale blubber.

and 9a). The accuracy of the mass of hormone measured from the amount added was linear in each assay (Figs 7b, 8b, and 9b).

b) Assay Validations for Humpgack Whales (Megaptera novaeangliae)

The EIA for progesterone, testosterone and cortisol were validated for humpback whale blubber (Figs 7, 8 and 9). The use of extracted blubber from both male and female humpback whales in each assay resulted in parallel displacement of the standard curves (Figs 7a, 8a,

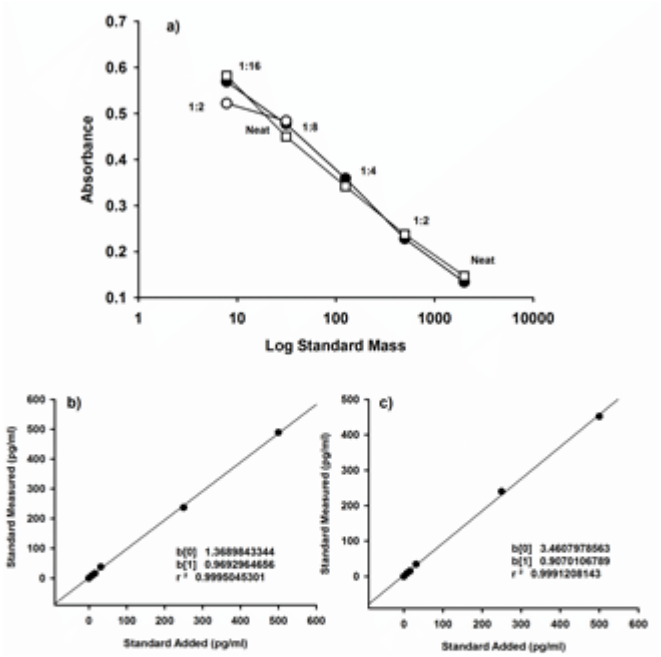

Fig 8. Enzyme immunoassay (EIA) validations for testosterone in humpback whale blubber. a) Parallel displacement of the standard curve (solid circles) measured by optical absorbance, using both female (open circles) and male (open squares) blubber. Accuracy of the assay with mass added (ng) reflecting the mass measured (ng) across the standard curve for b) female and c) male humpback whale blubber.

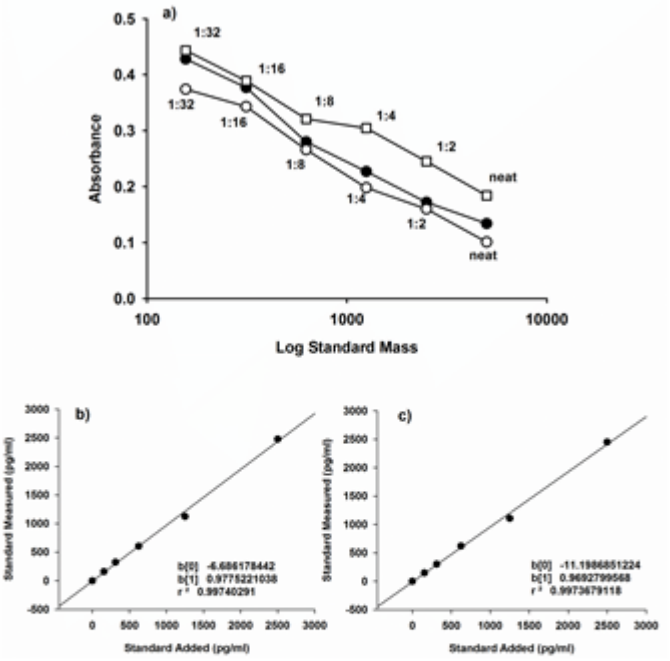

Fig 9. Enzyme immunoassay (EIA) validations for cortisol in humpback whale blubber. a) Parallel displacement of the standard curve (solid circles) measured by optical absorbance, using both female (open circles) and male (open squares) blubber. Accuracy of the assay with mass added (ng) reflecting the mass measured (ng) across the standard curve for b) female and c) male humpback whale blubber.

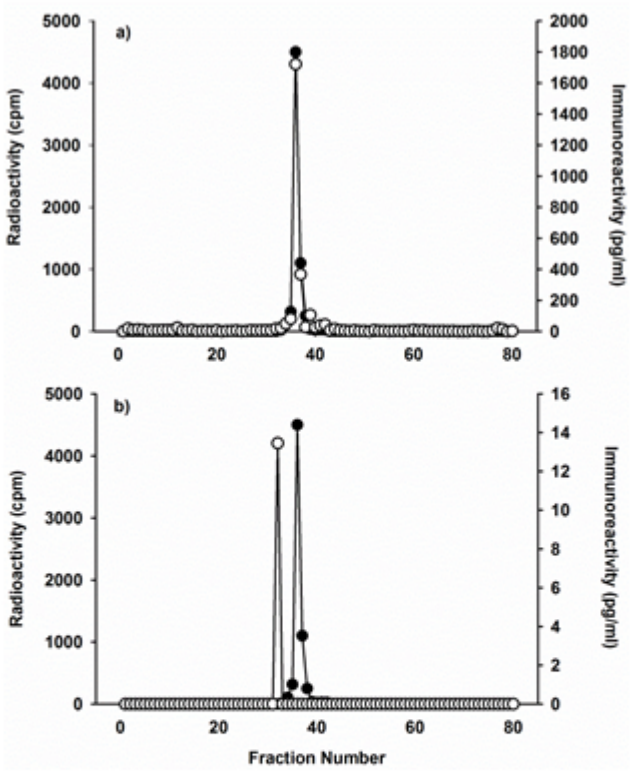

Fig 10. HPLC of progesterone in a) pregnant female and b) a non-pregnant female humpback whale. Filled circles represent tritiated progesterone (^{3}H-progesterone) tracer as a standard curve. Note that in a) 81% of immunoreactivity is associated with the ^{3}H-progesterone standard whereas in b) 100% of the immunoreactivity is a more polar metabolite in the non-pregnant female. A 1ml/min flow rate was used with a 32-50-75-100% acetonitrile/H_2O gradient.

HPLC was run for progesterone on both pregnant and non-pregnant blubber (Fig 10). Tritiated progesterone (^{3}H-progesterone) exhibited a defined peak between fractions 34-38 (Fig 10). In the pregnant female, 81% of the immunoreactive progesterone coeluted with ^{3}H progesterone (Fig 10a) whereas 100% of the immunoreactive progesterone in the non-pregnant whale was measured as a more polar (or conjugated) form of progesterone (Fig 10b).

Depth studies were conducted to ascertain the uniformity of steroid hormones in various issues including skin, blubber and muscle. These results are currently being plotted and analyzed.

COMMUNICATION WITH OTHER ONR PIs

Several interactions have taken place with other ONR project PIs. We have communicated with Dr. Nick Kellar and are conducting a preliminary inter-laboratory collaboration with his lab using common dolphins. We have also drafted a preproposal to conduct a large scale inter-laboratory collaboration. We have communicated extensively with Drs Dan Crocker and Dorian Houser in completing the review on stress physiology in marine mammals (Atkinson et al. 2015). Recent communications with Dr. Tracy Romano on a beluga pubication are also occurring (see below).

IMPACT/APPLICATIONS

The validated assays will be used to analyze stress and sex steroid hormones to compare physiological function relative to reproductive state (Objective 2 of this project). We have also submitted a preproposal to ONR to develop the analysis relative to anthropogenic disturbance such as noise.

DISSEMINATION OF INFORMATION:
Presentations and Abstracts:

1) Atkinson, S. (2015) Detection of reproductive status in eastern North Pacific blue whales. International Whaling Commission Report, San Diego, CA. SC/66a IA-WP3. 3pp.
2) Atkinson, S., Straley, J., Pack, A., Gabriele, C., Gendron, D., Mashburn, K. (2015) Is assessing reproductive state a prerequisite for stress studies? In proceedings of the 21st Biennial Conference on the Biology of Marine Mammals. San Francisco, CA. Dec 13-18.
3) Cates, K., Atkinson, S., Moran, J., Pack, A., Straley, J. (2015) Do testosterone levels of humpback whales suggest breeding activity in Alaskan feeding grounds? In Proceedings of the 21st Biennial Conference on the Biology of Marine Mammals. San Francisco, CA. Dec 13-18.

Peer-reviewed publications:

1) Atkinson, S., Crocker, D., Houser, D., Mashburn, K. (2015) Stress physiology and behavior in marine mammals: How well do they fit the terrestrial model? J. Comp. Physiol. B. 185:463-486
2) Atkinson, S., Gendron, D., Mashburn, KL., Branch, TA., Brownell Jr. R.L. (2015) Determination of pregnancy rates and biomarkers from the blubber of eastern North Pacific blue whales. J. Cet. Res. Manag. (In prep)

RELATED PROJECTS

A master's candidate, Ms Kelly Cates, was recruited in 2014 to the University of Alaska Fairbanks (UAF), School of Fisheries and Ocean Sciences (SFOS) to study physiology of male humpback whales. Her project is on reproduction in male humpback whales. Her project has made substantial progress with sample collection, by piggy-backing onto existing field studies and by accessing archived samples from the NMFS Southwest Fisheries Science Center.

Another Ph.D. student has been admitted to UAF's SFOS, Ms Valentina Melica. Her funding is coming from the Fulbright International Fellowship Program. We are hoping to have her work on blue whale or another large whale stress physiology.

An additonal related project has been through an on-going study of free-ranging and captive beluga whales, for which multiple endocrine parameters have been measured. This work has recently been drafted as a manuscript for publication (Atkinson et al., In Prep).

REFERENCES

Atkinson, S., Arnould, J. P. Y., Mashburn, K. 2011. Plasma cortisol and thyroid hormone concentrations in pre-weaning Australian fur seal pups. Gen. Comp. Endocrinol. 172:277-281.

Atkinson, S., Combelles, C., Vincent, D., Nachtigall, P. and Pawloski, J. 1999. Monitoring of progesterone in captive female false killer whales, pseudorca crassidens. Gen. Comp. Endocrinol. 115:323-332.

Atkinson, S., Crocker, D., Houser, D., Mashburn, K. 2015. Stress physiology and behavior in marine mammals: How well do they fit the terrestrial model? J. Comp. Physiol. B. 185:463-486

Atkinson, S., Gendron, D., Mashburn, KL., Branch, TA., Brownell Jr. R.L. 2015. Determination of pregnancy rates and biomarkers from the blubber of eastern North Pacific blue whales. J. Cet. Res. Manag. (In prep).

Atkinson, S., Yoshioka, M. 2007. Endocrinology of Reproduction. In: Reproductive Biology and Phylogeny of Cetacea (B.G.M. Jamieson Ed.) Science Publishers, Inc. Enfield, New Hampshire 7:171-192.

Calkins, D.G., Atkinson, S., Mellish, J-A., Waite, J.N., Carpenter, J.R. 2013. The pollock paradox: Juvenile Steller sea lions experience rapid growth on pollock diets in fall and spring. J. Exp. Mar. Biol. Ecol. 441:55-61.

Di Poi, C., Atkinson, S., Hoover-Miller, A., Blundell, G. 2015. Maternal buffering of stress response in free-ranging Pacific harbor seal pups in Alaska. Mar. Mammal Sci. 31(3):1098-1117.

Ellsworth, A., Buck, C.L., Atkinson, S., Hollmen, T. 2014. Longitudinal monitoring of sex steroid hormones in excrement of spectacled eiders (*Somateria fisheri*).Gen. Comp. Endocrinol. 198:59-65.

Geiger, G.L., Atkinson, S., Waite, J.N., Blundell, G.M., Carpenter, J.R., Wynne, K. 2013. A new method to evaluate the nutritional composition of marine mammal diet from scats applied to harbor seals in the Gulf of Alaska. J. Exp. Mar. Biol. Ecol. 449: 118-128.

Greig, D.J., Mashburn, K.L., Rutishauser, M., Gulland, F.M.D., Willliams, T.M., Atkinson, S. 2007. Seasonal changes in circulating progesterone and estrogen concentrations in the California sea lion (*Zalophus californianus*). J. Mammal. 88(1):67-72.

Hutchinson, E., Atkinson, S., Burek Huntington, K. 2015. Growth and sexual maturation in male northern sea otters (*Enhydra lutris kenyoni*) from Gustavus, Alaska. J. Mammal. Doi:10.1093/jmammal.gyv114.

Jaatinen, K., Seltmann, M.W., Hollmen, T., Atkinson, S., Mashburn, K., Ost, M. 2013. Context dependency of baseline glucocorticoids as indicators of individual quality in a capital breeder. Gen. Comp. Endocrinol. 191: 231-238.

Keogh, M.J., Atkinson, S. 2015. Endocrine and immunological responses to adrenocorticotrophic hormone (ACTH) administration in juvenile harbor seals (*Phoca vitulina*) during winter and spring. Comp. Biochem. Phys. A: 188:22-31.

Keogh, M.J., Atkinson, S., Maniscalco, J.M. 2013. Body condition and endocrine profiles of Steller sea lion (*Eumetopias jubatus*) pups during the early postnatal period. Gen. Comp. Endocrinol. 184:42-50

Mashburn K.L, Atkinson, S. 2004. Evaluation of adrenal function in serum and feces of Steller sea lions (*Eumetopias jubatus*): influences of molt, gender, sample storage, and age on glucocorticoid metabolism. Gen. Comp. Endocrinol. 136:371-381.

Mashburn K.L., Atkinson, S. 2007. Seasonal and predator influences on adrenal function in adult Steller sea lions: Gender matters. Gen. Comp. Endocrinol. 150:246-252.

Mashburn, K.L., Atkinson, S. 2008.. Variability in leptin and adrenal response in juvenile Steller sea lions (*Eumetopias jubatus*) to adrenocorticotropic hormone (ACTH) in different seasons. Gen. Comp. Endocrinol. 155(2):352-385.

Mellish, J., Hennen, D., Thomton, J., Petrauskas, L., Atkinson, S. Calkins, D. 2007. Permanent marking in an endangered species: Physiological response to hot-branding in juvenile Steller sea lions (*Eumetopias jubatus*). Wildl. Res. 34:43-47.

Myers, M. Litz, B. Atkinson, S. 2009. The effects of age, season and geographic region on circulating serum cortisol concentrations in threatened and endangered Steller sea lions (*Eumetopias jubatus*). Gen. Comp. Endocrinol. 165:72-77.

Nilsson, P., Hollmén, T., Atkinson, S., Mashburn, K., Tuomi, P., Esler, D., Mulcahy, D. Rizzolo, D. 2008.. Effects of ACTH, capture, and short term confinement on glucocorticoid concentrations in harlequin ducks (*Histrionicus histrionicus*). Comp. Biochem. Physiol. 149(1):275-283.

Oki, C. Atkinson, S. 2004. Diurnal patterns of cortisol and thyroid hormones in the harbor seal (*Phoca vitulina*) during summer and winter seasons. Gen. Comp. Endocrinol. 136:289-297.

Petrauskas, L., Atkinson, S., Gulland, F., Mellish, J.A. Horning, M. 2008. Monitoring glucocorticoid response to rehabilitation and research procedures in California and Steller sea lions. J. Exper. Biol. 309A:73-82.

Petrauskas, L., Tuomi, P., Atkinson, S. 2006. Noninvasive monitoring of stress hormone levels in a female Steller sea lion (*Eumetopias jubatus*) pup undergoing rehabilitation. J. Zoo Wildl. Med.37 (1):75-78.

Petrauskas, L.R. Atkinson, S. 2006. Variation of fecal corticosterone concentrations in captive Steller sea lions (*Eumetopias jubatus*) in relation to season and behavior. Aqua. Mamm. 32(2):168-174.

Pietraszek J, Atkinson, S. 1994. Concentrations of estrone sulfate and progesterone in plasma and saliva, vaginal cytology, and bioelectric impedance during the estrous cycle of the Hawaiian monk seal (*Monachus schauinslandi*) Mar. Mammal Sci. 10(4): 430-441.

Seltmann, M.W., Ost, M., Jaatinen, K., Atkinson, S., Mashburn, K., Hollmen, T. 2012. Stress responsiveness, age and condition interactively affect risk taking in female eiders. Anim. Behav. 84:889-896.

Trumble, S.J., O'Neil, D.O., Cornick, L.A., Gulland, F., Castellini, M.A., Atkinson, S. 2013 Endocrine changes in harbor seal (*Phoca vitulina*) pups undergoing rehabilitation. Zoo Biol. 32:134-141.

Verrier, D., Atkinson, S., Guinet, C., Groscolas, R., Arnould, J.P.Y. 2012. Hormonal responses to extreme fasting in subantarctic fur seal (*Arctocephalus tropicalis*) pups. Am. J. Physiol-Reg I. 302: R929-R940.

Villegas-Amtmann, S., Atkinson, S. Costa, D.P. 2009. Low synchrony in the breeding cycle in Galapagos sea lions revealed by seasonal progesterone concentrations. J. Mammal. 90(5):1232-1237.

Villegas-Amtmann, S., Atkinson, S. Costa, D.P. 2012. Seasonal variation in muscle oxygen stores attributed to diving behavior, environmental temperature and pregnancy in a marine predator the California sea lion. Comp. Biochem. Physiol. A. 162:413-420.

Wang, D., Shelver, W.L., Atkinson, S., Mellish, J.A., Li, Q.X. 2010. Tissue distribution of polychlorinated biphenyls and organochlorine pesticides and potential toxicity to Alaskan northern fur seals assessed using PCBs congener specific mode of action schemes. Arch. Environ. Contam. Toxicol. 58:478-488.

West, K.L., Atkinson, S., Carmichael, M.J., Sweeney, J.C., Krames, B., Krames, J. 2000. Concentrations of progesterone in milk from bottlenose dolphin during different reproductive status. Gen. Comp. Endocrinol.117:218-224.